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Simultaneous Extraction and Determination of 5-Hydroxy tryptophol and 5-Hydroxyindol-3-acetic Acid in Urine of Alcoholism by HPLC with Fluorescence Detection

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5-Hydroxy tryptophol (5-HTOL) and 5-hydroxyindol-3-acetic acid (5-HIAA), two serotonin metabolite, were derivatized with benzylamine at room temperature in a mixture of 0.3 M 3-cyclohexylamino-1-propane sulfonic acid (CAPS buffer, pH 12) and methanol in presence of potassium hexacyanoferrate(III), which produce highly fluorescent benzoxazole derivative. The resulting species were separated on XDB C_{18} (150 mm × 4.6 mm, 5 μm) with isocratic elution using a mixture of methanol and 15 mM acetate buffer (pH 4.5) containing 1 mM octansulfonic acid and acetonitrile as modifier (65:35). They were detected spectroflourometrically at 480 nm with excitation wavelength of 345 nm. Urine samples of ethanol positive (alcoholism) were collected from 20 persons sent to forensic medical center toxicology laboratory, Tehran, Iran. Sixty urine samples of ethanol negative persons were also collected. 5-Hydroxy tryptophol and 5-hydroxyindol-3-acetic acid contents were determined after liquid-liquid extraction and derivitazation. The mean value of 5-HTOL/5-HIAA ratio for ethanol positive and negative samples was 74.25 and 2.19 pmol/nmol, respectively. The cut off value was 10.7 pmol/nmol.

Key Words: Serotonin metabolism, 5-HTOL/ 5-HIAA, Alcoholism, Urine analysis, HPLC.

INTRODUCTION

Ethanol analysis, most commonly accomplished by headspace gas chromatography (GC), is one of the most common and routine tests performed on forensic specimens. Recently, possible exploitation of the metabolism of serotonin as a biological marker for ethanol consumption has begun to gain interest in the field of forensic science[1]. Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine commonly found in nature[2]. In humans, 5-HT is found throughout the body, with substantial concentrations found in the gastrointestinal tract and blood platelets[3]. The metabolism of 5-HT initially involves oxidative deamination to form the intermediate aldehyde, 5-hydroxyindole-3-acetaldehyde (5-HIAL). Oxidation of the aldehyde, catalyzed by aldehyde dehydrogenase, leads to formation of 5-hydroxyindole-3-acetic acid (5-HIAA), the predominant metabolite of 5-HT[3,4]. Reduction, catalyzed by aldehyde

reductase, leads to formation of 5-hydroxy tryptophol (5-HTOL), a relatively minor metabolite of 5-HT[1]. However, ethanol consumption has been shown to lead to a significantly enhanced production of 5-HTOL. An increase in 5-HTOL concentration following ethanol consumption was first reported in 1967. Since that time it has been clearly demonstrated that consumption of ethanol shifts 5-HT metabolism to promote formation of 5-HTOL and, some reports indicate, to reduce the formation of 5-HIAA[1,5-7].

Different methods have been proposed for simultaneous determination of 5-HT, noradernaline, dopamine and their metabolites, namely high performance liquid chromatography with electrochemical (LC-ED) or fluorescence (LC-FL)[8-16] detection. Gas chromatography and mass spectrometry (GC-MS) was also used after derivitization[17,18]. For determination of 5-HTOL/5-HIAA ratio, levels of 5-HTOL and 5-HIAA in individual samples are different and have been measured using two completely different analytical techniques. 5-Hydroxyindole-3-acetic acid concentrations are typically measured at sub-nanomolar levels using liquid chromatography with electrochemical detection (LC-EC)[19,20]. 5-Hydroxy tryptophol is also accessible by LC-EC, but the detection limits are typically insufficient to measure this compound in most pertinent biological specimens. For this reason, 5-HTOL has typically been analyzed using gas chromatography with mass spectrometric detection (GC-MS)[21]. The employment of two different analytical techniques to obtain the 5-HTOL/5-HIAA ratio in a specimen obviously decreases the precision and reliability of the final result. In another study that two metabolites are measured by the same technique the 5-HTOL and 5-HIAA are extracted from urine by two different extraction conditions[17].

In this study simultaneous extraction and determination of 5-HTOL and 5-HIAA in urine of alcoholism sent to forensic medical center toxicology laboratory was evaluated. Finally the cut off value of 5-HTOL/5-HIAA was calculated from the results.

EXPERIMENTAL

All chemicals were purchased in the highest possible purity and used without further purification. Sodium chloride, sodium acetate, acetic acid, potassium hexa cyanoferrate, methanol and acetonitrile both HPLC grade and other solvents used in this study were purchased from Merck, Germany. Octansulphonic acid, β-glucuronidase, 5-hydroxyindol-3-acetic acid and 5-hydroxy tryptophol were purchased from sigma (St. Louis, MO). Doubly distilled water was used throughout the study.

Chromatography was performed on Shimadzue (Japan) high performance liquid chromatograph equipped with SPD 10AVP fluorescence detector, DGU-14A degasser, LC-10ADVP pump, FCL-10ALVP flow controller and SCL-10AVP system controller and LC lab solution software. Separation was done on XDB-C_{18} (150 mm × 4.6 mm, 5 μm) equipped with guard column of the same packing (Agilent technology). 20 μL injection loops was used. Excitation wavelength was set at 345 nm and emission was reported at 480 nm.

Mobile phase: 450 mL of 15 mM acetate buffer (pH 4.5) containing 1 mM octansulfonic acid was mixed with 50 mL acetonitrile. This solution referred to as aqueous solution. Methanol and aqueous solution was mixed in 65:35 ratios and used as mobile phase.

Stock solutions of 2.63 mmol L^{-1} of 5-HIAA and 5.64 mM 5-HTOL were prepared by transferring 10 mg of each compound separately to 10 mL volumetric flask and diluted to the mark with methanol. The working solutions in the range of 1-10 pmol mL^{-1} were freshly prepared on the day of use with step by step dilution.

Sample collection: Ethanol positive samples were collected from 20 persons sent to forensic medical center of Tehran (Iran) and ethanol negative samples were collected from 16 nonalcoholic volunteers. The samples were stored at -20 ºC before analysis. Each person filled a questionnaire indicating the kind of drug and food they used.

Enzyme preparation: Since 5-HTOL in human-derived specimens is predominately found as the glucuronide derivative, β-glucuronidase was initially used to hydrolyze the samples. A solution of β-glucuronidase was prepared by adding 2.5 mL of 0.10 mM sodium acetate buffer (pH 5) to 250000 units of the solid enzyme and mixed to dissolve completely. The final concentration of 100000 units/mL was achieved. This solution was stored at -20 ºC and used within 7 days.

Extraction technique: The urine samples were thawed at room temperature in a dark place for about 2 h. Then they were centrifuged at 5000 rpm for about 10 min. 1 mL of supernatant was transferred to 16 mm × 150 mm culture tubes. To each sample, 25 µL (2500 unit) β-glucuronidase solution and 1 mL of 0.10 mM sodium acetate buffer (pH 6) was added. The samples were vortexed briefly and incubated at 70 ºC for 1 h to hydrolyze the glucuronide conjugate. Following hydrolysis, samples were allowed to cool to room temperature. Then, 2.00 mL of a 0.10 M sodium acetate buffer (pH 6) and 0.50 mL of a saturated sodium chloride solution were added and the tubes were briefly vortexes. Ethyl acetate (9 mL) was added to each tube and the tube was tightly capped. The mixture was then placed on a rotary mixing wheel and gently mixed for 20 min by simple rotation of the wheel at 6 rpm. Following mixing, the samples were centrifuged at 820 × g for 5 min. The organic (upper) layer of each sample was transferred using a disposable pipette to a clean 10 mL conical tube and dried in a water bath at 40 ºC under a constant stream of nitrogen. The dried samples were removed from the evaporator and 80 ml of methanol was added.

Derivatization method: Derivatization was performed according to the reported method[22]. All standards and extracted samples were derivatized by this procedure. So, 20 µL of benzylamine derivatization reagent at pH 12 was added to 20 µL of each standard and sample solutions placed in a 300 µL micro vial. After standing at room temperature for about 2 min the samples were injected onto the column and the resulting chromatograms were recorded.

RESULTS AND DISCUSSION

HPLC Conditions: HPLC optimum conditions were obtained using standard solutions containing mixtures of 5-HTOL and 5-HIAA. The best separation was achieved using methanol: acetate buffer (pH 4.5) contains acetonitrile and 1 mM of octansulfonic acid as modifier (65:35) at flow rate of 0.6 mL min^{-1}. A typical chromatogram is shown in Fig. 1. The retention time of 5-HTOL and 5-HIAA were reported as 15 and 10 min, respectively.

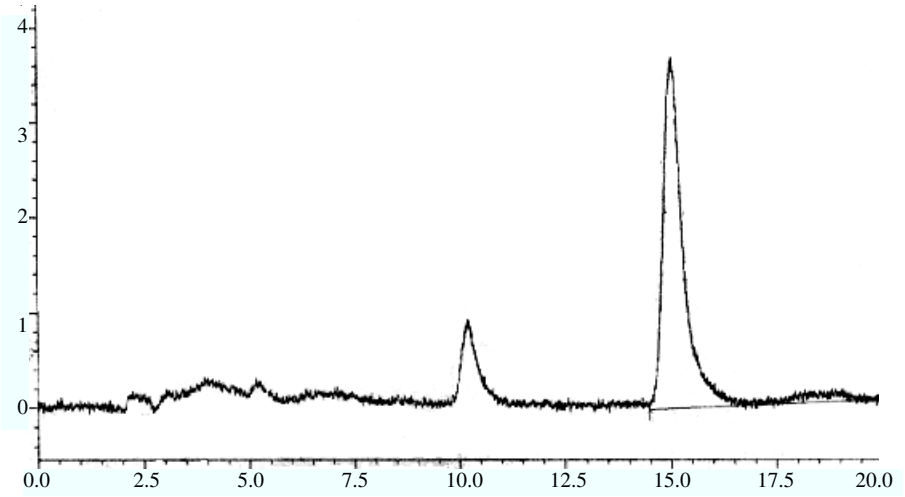

Fig. 1. Chromatogram of 10 pmol mL^{-1} of both 5-HTOL and 5-HIAA. Conditions: XDB C_{18} (150 mm × 4.6 mm, 5 µm) equipped with the same guard column. 20 µL injection. Excitation wavelength of 345 nm and emission wavelength of 480 nm

Validation of the method: The calibration curve, relation between peak area and concentration of standards, were linear in the range of 1-10^5 pmol mL^{-1} for 5-HTOL ($y = 0.3124x + 5.7837$, $R^2 = 0.9982$) and 10-10^5 pmol mL^{-1} for 5-HIAA ($y = 0.3126x + 5.7927$, $R^2 = 0.9976$). A typical chromatogram of standards is presented in Fig. 1. The detection limit, S/N ratio of three, was 10 and 2.5 fmole per 20 µL injection for 5-HIAA and 5-HTOL, respectively. Between days and within days coefficients of variation were also within acceptable range (Table-1). The accuracy of the method was evaluated by spiking different amount of standards to samples before extraction, then determining the amount added using the calibration curve after derivatization. The results are shown in Table-2. As the results indicate the recovery for 5-HIAA is better than 5-HTOL.

Cutoff value: Four ethanol positive samples were omitted because of loss of information contain in questionnaire. Typical chromatograms of ethanol positive and negative samples are presented in Fig. 2. As it is expected the peak of 5-HTOL is increased following alcohol consumption[18]. The results of 5-HTOL/5-HIAA ratio

TABLE-1
WITHIN DAYS AND BETWEEN DAYS VARIATION OF THE RESULTS
OBTAINED BY THE METHOD (SEE TEXT FOR DETAILS)

5-HIAA pmol/mL	CV		5-HTOL (pmol/mL)	CV	
	Within	Between		Within	Between
10	5.91	7.12	10	8.05	10.70
100	5.73	3.72	100	5.67	9.25
1000	3.03	5.12	1000	4.90	4.50

TABLE-2
RECOVERY OF THE METHOD FOR THE SIMULTANEOUS LIQUID-LIQUID
EXTRACTION AND DETERMINATION OF 5-HTOL AND 5-HIAA.
RESULTS ARE THE MEAN OF THREE MEASUREMENTS

Sample	5-HIAA pmol/mL		5-HTOL pmol/mL		Recovery (%)	
	Added	Recovered	Added	Recovered	5-HIAA	5-HTOL
1	9	6.30	9	4.91	70.00	54.56
2	93	65.60	98	56.56	70.54	57.71
3	981	747.40	933	555.00	76.19	59.49

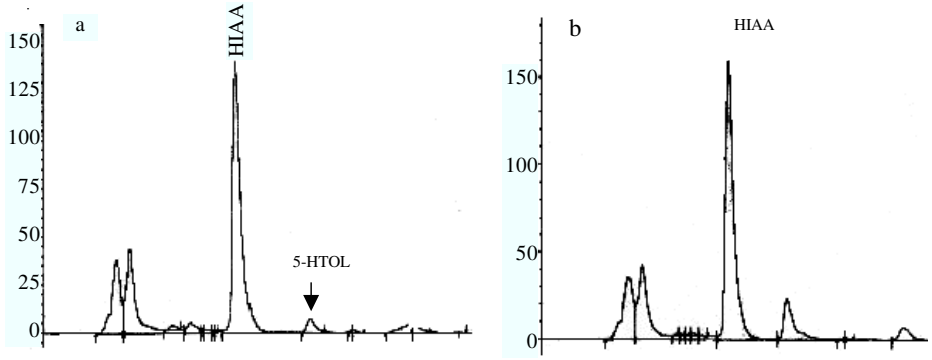

Fig. 2. A typical chromatogram for extracted urine samples (control negative, (b) control positive (alcoholism). Conditions: XDB C_{18} (150 mm × 4.6 mm, 5 μm) equipped with the same guard column. 20 μL injection. Excitation wavelength of 345 nm and emission wavelength of 480 nm

for all samples are graphically presented in Fig. 3. The figure showed that the level of 5-HTOL/5-HIAA of ethanol negative samples does not change considerably but that of ethanol positive showed considerable deviation. This results are in good agreement with another finding in researches on some group who intentionally drank different amount of alcohol and after some specific times the 5-HTOL/5-HIAA were measured[23]. Since it is intended to judge that a person drinks alcohol the cut off value of 5-HTOL/5-HIAA is determined by 5 fold of mean value of 5-HTOL/5-HIAA obtained for ethanol negative samples[18]. So the cut off value were 10.7 pmol/nmol (Table-3). The reported values were in the range of 12-15 pmol/nmol[18].

TABLE-3
5-HTOL/5-HIAA OF ETHANOL NEGATIVE AND POSITIVE SAMPLES

Sample		5-HIAA (nmol/mL)	5-HTOL (pmol/mL)	5-HTOL/5-HIAA (pmol/mL)
Ethanol negative	Max	412.70	989.20	8.25
	Min	28.20	24.50	0.25
	Mean	190.23	372.03	2.19
	SD	114.00	315.83	2.14
Ethanol positive	Max	76.50	7112.70	227.00
	Min	12.90	728.80	16.00
	Mean	40.34	2481.90	74.25
	SD	16.21	2021.49	71.27

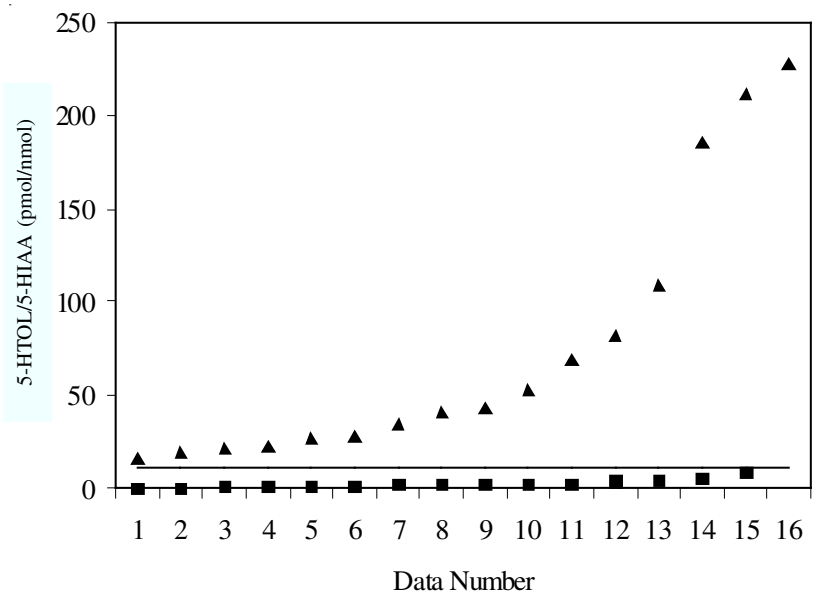

Fig. 3. Ratio of 5-HTOL/5-HIAA in ethanol positive △ and ethanol negative □ urine samples. The solid line showed the cut off value

Conclusion

As it is obvious from the results that this method can be used for simultaneous determination of 5-HTOL and 5-HIAA in urine of alcoholism. The ethanol concentrations of ethanol positive samples were determined by gas chromatography in forensic center laboratory. Fig. 4 shows the correlation between the 5-HTOL/5-HIAA and EtOH concentrations. As it is also reported in other studies[24] the 5-HTOL/5-HIAA increase as EtOH decreases. The increase in this ratio is due to increase in 5-HTOL concentration in urine following the consumption of alcohol. It was also reported that 5-6 h after alcohol intake rise in concentration of 5-HTOL occurs and 5-HTOL/5-HIAA ratio remains elevated for several hours after ethanol is no longer detectable.

The results also showed that with such low extraction efficiency the difference in 5-HTOL/5-HIAA ratio of alcoholisms and nonalcoholic persons are obviously observed. So this method can be used in forensic toxicology laboratories. In order to obtain higher extraction efficiency our research groups are working on simultaneous solid phase extraction of 5-HTOL and 5-HIAA using nanosorbents.

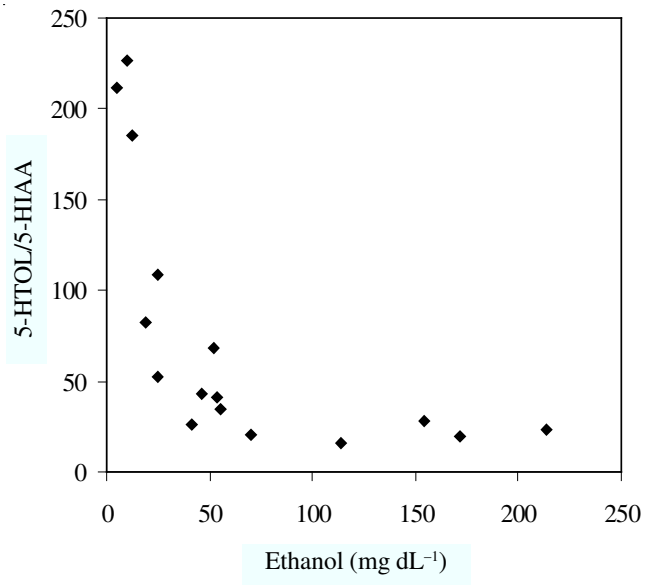

Fig. 4. Correlation between ethanol uptake and the 5-HTOL/5-HIAA ratio

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